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Note

High-performance liquid chromatography of 4-nitrophenyl organophosphinates and chiral-phase separation of enantiomers

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Certain 4-nitrophenyl organophosphinates are rapid, transient inhibitors of acetylcholinesterase[1] and carboxylesterase[2–3]. Stereospecificity of these reactions has not been investigated. While enantiomers of aryl hydroxy phosphonates have been separated directly by chiral-phase chromatography[4], organophosphinate enantiomers have not been resolved directly, but only after derivatization to diastereomers[5]. We report the direct chromatographic separation of several 4-nitrophenyl organophosphinate enantiomers on a commercially available chiral-phase column as well as the normal-phase and reversed-phase high-performance liquid chromatography (HPLC) of four diverse series of these compounds. These techniques are useful for preparing small quantities of pure enantiomers and also for detecting organophosphinates in extracts of biological samples.

MATERIALS AND METHODS

Organophosphinates were provided by the Institute of Chemical Defense of the U.S. Army Medical Research and Development Command as crystalline samples of 99% purity. Samples of 4-nitrophenol and diethyl-4-nitrophenylphosphate (paraoxon) were purchased from Sigma (St. Louis, MO, U.S.A.). Unless specified differently, all chemicals were prepared as acetonitrile solutions. Acetonitrile, dichloromethane (Fisher Scientific, Pittsburgh, PA, U.S.A.), water, 2-propanol and *n*-hexane (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were HPLC grade.

The instrument consisted of a Tracor 980A solvent programmer (Tracor Instruments, Austin, TX, U.S.A.), a Tracor 950 high-pressure pump, a Valco injector with 10-μl loop, a water jacket (Alltech Assoc., Chicago, IL, U.S.A.) with Neslab RTE-9 circulator bath (Neslab Instruments, Portsmouth, NH, U.S.A.) for control of column temperature, a Tracor 970A ultraviolet detector, and a Fisher strip-chart recorder. Organophosphinates were detected by absorbance at 270 nm and retention times were measured from the time of injection. The limit of detection for most compounds was less than 20 ng; 2 μg of each organophosphinate were injected.

Normal-phase HPLC was performed using a 10-μm Partisil® 25 cm × 4.6 mm I.D. column held at 36°C preceded by a 30–38 μm Pellosil® 6 cm × 4.6 mm I.D. guard column (Whatman, Clifton, NJ, U.S.A.). Solvents were degassed by sonication for 3 min, then mixed by the programmer and pumped isocratically. Acetonitrile

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solutions of samples were diluted 1:19 with 2-propanol–hexane (20:80) prior to injection.

Reversed-phase HPLC was performed on a 10-μm Lichrosorb® RP-8 25 cm × 4 mm I.D. column at ambient temperature with a 30–40 μm Perisorb® RP-8 2.5 cm × 4 mm I.D. guard column (E. Merck, Darmstadt, F.R.G.). Solvents were mixed manually, degassed and pumped isocratically.

Chiral-phase chromatography was performed using a 5-μm Bakerbond™ 25 cm × 4.6 mm I.D. column (J. T. Baker, Phillipsburg, NJ, U.S.A.) held at 18°C and preceded by the Whatman Pellosil guard column described previously. Solvents were mixed manually, degassed and pumped isocratically. For preparative HPLC, up to 250 μg of each chiral organophosphinate were injected repeatedly, the lesser retained enantiomer was collected to its peak, and the greater retained enantiomer was collected following its peak.

Mice (CF-1®, Charles River Breeding Labs., Wilmington, MA, U.S.A.) of approximately 40 g each were injected in leg muscle with 4-nitrophenyl diphenylphosphinate in polyethyleneglycol 200 (Sigma) with 10% dichloromethane and euthanized with diethyl ether after 2 min. Legs were excised, skinned, covered with dichloromethane, frozen with liquid nitrogen and stored at −5°C. Thawed legs were homogenized in dichloromethane over 10 g sodium sulfate in a mortar and pestle, then extracted by Soxhlet for 10 h. Extracts were concentrated to 0.5 ml *in vacuo* and transferred quantitatively to a 10-ml volume in 0.01% acetic acid in methanol. Samples were filtered through a 0.45-μm nylon membrane (Rainin, Woburn, MA, U.S.A.) and reversed-phase HPLC was performed as described previously. Data were acquired for quantitation on an IBM Instruments 9000 computer with chromatography application program CAPS2 (IBM Instruments, Danbury, CT, U.S.A.). The limit of detection by HPLC was less than 0.12 μg and the detector response was linear to more than 1.0 μg.

Rabbit serum, to which 4-nitrophenyl diphenylphosphinate had been added at 0.1 mg/ml, was extracted by adding 0.1 ml to 2 ml ethyl acetate containing 0.2% glacial acetic acid. This was mixed with 2 ml water and the ethyl acetate layer was collected, followed by two additional ethyl acetate extractions. Ethyl acetate was evaporated by nitrogen stream and the residue redissolved in 2 ml warm acetonitrile. Acetonitrile was partitioned against hexane three times, discarding the hexane layers. The acetonitrile was concentrated to 0.1 ml and a 10 μl sample was injected on the reversed-phase column.

RESULTS AND DISCUSSION

Retention times of organophosphinates on silica were directly related to polarity of the molecules as observed when 2-propanol–hexane (10:90) was used to elute compounds of the phenyl series, halogen series and bis-substituted series (Table I). The common decomposition product, 4-nitrophenol, and the organophosphate, paraoxon, were retained 9.8 and 12.8 min, respectively. Since compounds of the methyl series were absorbed strongly, it was necessary to increase the elutropic power of the mobile phase by doubling the 2-propanol content so that they were retained less than 20 min. Then 4-nitrophenol and paraoxon were eluted in 5.5 and 8.2 min, respectively.

TABLE I

NORMAL-PHASE HPLC OF 4-NITROPHENYL ORGANOPHOSPHINATES ON SILICA COLUMN

Mobile phase: 2-propanol–hexane. Flow-rate: 1 ml/min. Column temperature: 36°C.

Series	*P–C Bonded substituents*		*Retention time (min ± S.E.)*	*n*	*% 2-propanol in mobile phase*
	R_1	R_2			
Methyl	CH_3	1-Naphthyl	12.80 ± 1.05	4	20
	CH_3	2-Thienyl	18.45 ± 1.20	4	20
	CH_3	Phenyl	19.30 ± 0.941	6	20
Phenyl	$(CH_3)_2CH$	Phenyl	9.26 ± 0.265	7	10
	CH_3CH_2	Phenyl	15.52 ± 0.693	8	10
	CH_3	Phenyl	>30	2	10
Halogen	CCl_3	Phenyl	5.71 ± 0.125	7	10
	$CHCl_2$	Phenyl	7.73 ± 0.242	7	10
	CH_2Cl	Phenyl	15.07 ± 0.747	6	10
Bis-substituted	Phenyl	Phenyl	5.6	1	20
			9.68 ± 0.717	12	10
	2-Thienyl	2-Thienyl	12.42 ± 0.802	9	10
	2-Furyl	2-Furyl	13.18 ± 0.626	8	10

Retention times of organophosphinates on the octylsilyl-bonded column were inversely related to polarity of the compounds and inversely correlated with retention times on silica (Tables I and II). On the octylsilyl column, all organophosphinates were resolved from 4-nitrophenol which was retained 4.2 min in acetonitrile–water (50:50); paraoxon was eluted in 6.6 min. Resolution of methyl series compounds from each other was satisfactory by reversed-phase chromatography (Fig. 1) while these compounds were not resolved by normal-phase chromatography.

As indicated by HPLC, the most polar of these organophosphinates was the methyl(2-furyl) compound while the least polar was the trichloromethyl(phenyl) compound. In the halogen series, the addition of chlorine atoms dramatically influenced chromatographic behavior while there was a similar effect when the aliphatic substituent was enlarged in the phenyl series. Changes from aryl to heteroaryl substituents had less effect on the chromatographic characteristics of the organophosphinates.

Separation of enantiomers was observed for racemic mixtures of each of three chiral organophosphinates on the ionically bonded, chiral-phase column (Table III). Elution on this column was in the same order as in normal-phase; *i.e.*, the methyl series compounds were most highly retained. Since this column degenerates in polar mobile phase, enantiomers of the chiral, methyl series compounds could not be separated. Achiral, bis-substituted organophosphinates chromatographed as single peaks (Table III). Chiral separations were enhanced by decreasing the polarity of the mobile phase and also by decreasing the column temperature to 18°C.

Enantiomers of 4-nitrophenyl isopropyl(phenyl)phosphinate were isolated by collecting the eluate from baseline to the point of greatest absorbance of first peak,

TABLE II

REVERSED-PHASE HPLC OF 4-NITROPHENYL ORGANOPHOSPHINATES ON OCTYLSILYL BONDED COLUMN

Mobile phase: acetonitrile–water. Flow-rate: 1 ml/min. Column temperature: 24°C.

Series	*P–C Bonded substituents*		*Retention time (min ± S.E.)*	*n*	*% acetonitrile in mobile phase*
	R_1	R_2			
Methyl	CH_3	1-Naphthyl	8.81 ± 0.071	4	50
	CH_3	Phenyl	5.35 ± 0.062	4	50
			8.34 ± 0.075	2	40
	CH_3	2-Thienyl	5.18 ± 0.091	4	50
			7.54 ± 0.040	2	40
	CH_3	2-Furyl	4.62 ± 0.024	4	50
			6.62 ± 0.005	2	40
Phenyl	$(CH_3)_2CH$	Phenyl	8.08 ± 0.082	5	50
	CH_3CH_2	Phenyl	6.55 ± 0.093	5	50
	CH_3	Phenyl	5.35 ± 0.062	4	50
Halogen	CCl_3	Phenyl	17.37 ± 0.091	6	50
	$CHCl_2$	Phenyl	10.39 ± 0.056	7	50
	CH_2Cl	Phenyl	7.24 ± 0.038	7	50
	CH_3	4-CF_3 phenyl	9.13 ± 0.051	5	50
	(second peak)		(35.82 ± 0.350)		
Bis-substituted	Phenyl	Phenyl	11.18 ± 0.142	5	50
	2-Thienyl	2-Thienyl	9.17 ± 0.111	5	50
	2-Furyl	2-Furyl	6.98 ± 0.039	4	50
	CH_2Cl	CH_2Cl	5.33 ± 0.058	4	50

discarding the middle portion, and then collecting from the point of greatest absorbance of the second peak to baseline; accumulated collections were rechromatographed on the next day to observe the partially purified enantiomers (Fig. 2). When enantiomers of the ethyl(phenyl) compound were collected, accumulation from 22 injections of 0.05 mg each yielded 0.19 mg of the lesser retained enantiomer and 0.40 mg of the greater retained enantiomer. Concentration and rechromatography of these fractions indicated that the first peak was approximately 95% pure while the second peak was only 80% pure. The second peak was purified further by repeating the process.

Recovery of 4-nitrophenyl diphenylphosphinate following intramuscular injection of mice was 83.1% (± 14.3 S.E., $n = 5$) when doses ranged from 0.625 mg to 2.5 mg per mouse. Clean-up of samples was not necessary since the phosphinate was retained for 10 min and no other peaks were observed between 5 and 20 min. No phosphinate was observed in a control mouse injected with vehicle only. The recovery of phosphinate plus 4-nitrophenol from rabbit serum spiked with 4-nitrophenyl diphenylphosphinate was 80.0% (± 3.6 S.E., $n = 4$). There were no interfering

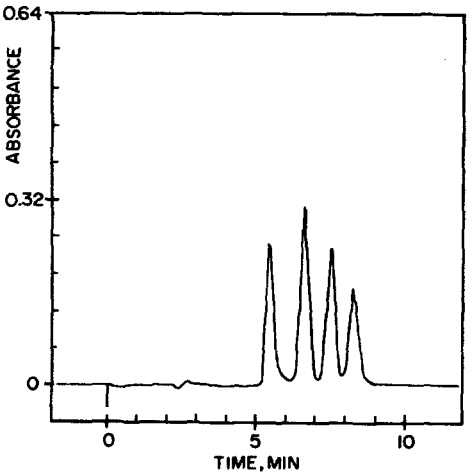

Fig. 1. Reversed-phase chromatography of 4-nitrophenyl organophosphinates on an octylsilyl column with acetonitrile–water (40:60) as the mobile phase; detection at 270 nm. Peaks in order of elution are 4-nitrophenol, 4-nitrophenyl methyl(2-furyl)phosphinate, 4-nitrophenyl methyl(2-thienyl)phosphinate and 4-nitrophenyl methyl(phenyl)phosphinate.

TABLE III

CHIRAL-PHASE HPLC OF 4-NITROPHENYL ORGANOPHOSPHINATES ON (*R*)-N-(3,5-DINITROBENZOYL)PHENYLGLYCINE-IONICALLY BONDED COLUMN

Mobile phase: 2-propanol–hexane. Flow-rate: 1 ml/min. Column temperature: 18°C.

Series	*P–C Bonded substituents*		*Retention time (min ± S.E.)*		*n*	*% 2-propanol in mobile phase*
	R_1	R_2	*1st enantiomer*	*2nd enantiomer*		
Methyl	CH_3	1-Naphthyl	> 47	–	2	10
Phenyl	$(CH_3)_2CH$	Phenyl	16.67 ± 0.048	17.18 ± 0.062	4	10
			26.40 ± 0.210	27.54 ± 0.226	4	5
	CH_3CH_2	Phenyl	20.06 ± 0.205	20.54 ± 0.219	4	10
			33.53 ± 0.208	34.57 ± 0.229	5	5
Halogen	$CHCl_2$	Phenyl	19.15 ± 0.108	19.55 ± 0.099	7	10
			29.92 ± 0.245	30.77 ± 0.244	4	5
	CH_2Cl	Phenyl	> 50	–	2	10
Bis-substituted	Phenyl	Phenyl	28.12 ± 0.129	–	4	10
			48.48 ± 0.258	–	4	5
	2-Thienyl	2-Thienyl	33.53 ± 0.176	–	7	10
			55.71 ± 0.292	–	2	5

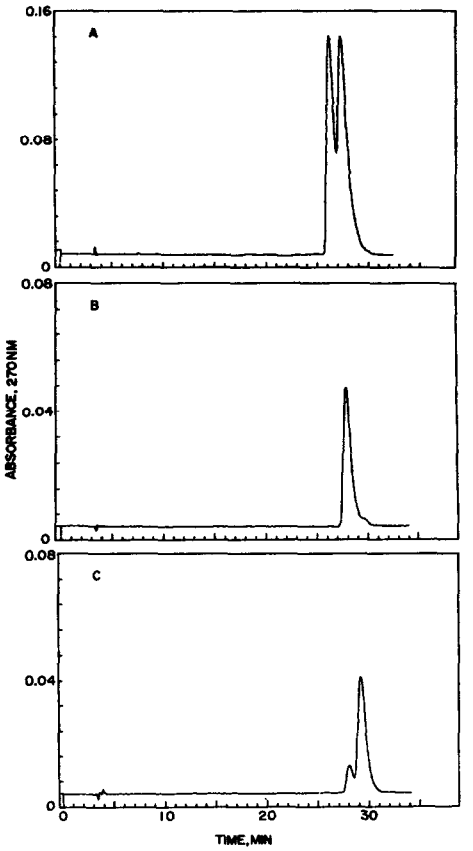

Fig. 2. Chiral-phase separation of enantiomers of 4-nitrophenyl isopropyl(phenyl)phosphinate; (A) chromatogram of racemic mixture; (B) chromatogram of lesser retained enantiomer; (C) chromatogram of greater retained enantiomer. Enantiomers in B and C were isolated using preparative liquid chromatography (see text).

peaks in the serum extracts. The actual limit of detection was not approached in this study and it appeared that phosphinate metabolism could be examined by this method.

Several HPLC methods were applied to organophosphinates; the chiral-phase separation of organophosphinate enantiomers should be particularlly useful for preparation of small quantities to be tested as enzyme substrates or inhibitors. Organophosphinates were readily chromatographed from biological samples.

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